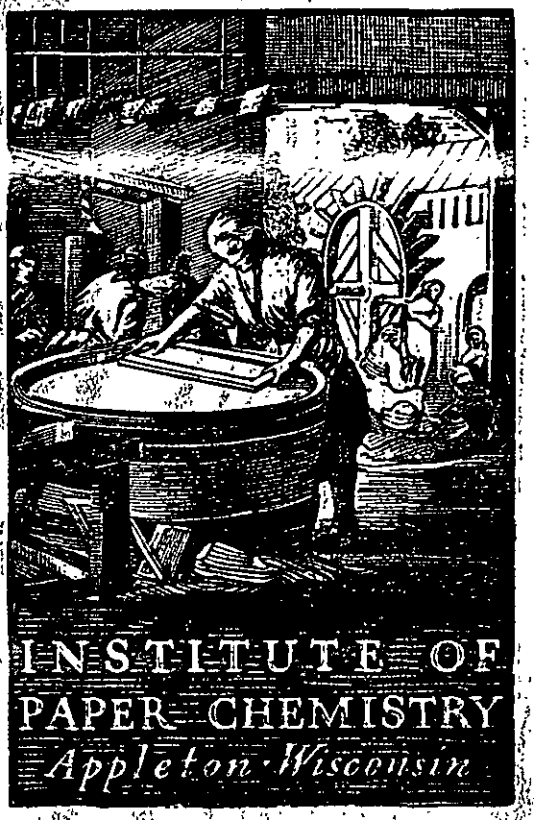

DEFINED MEDIA FOR ASPEN TISSUE CULTURE

Project 2351

Report Six

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

October 12, 1966

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

Hard white and vigorous tissue of triploid quaking aspen (Populus tremuloides) was maintained on Wolter's revised chemically defined medium, made with a low concentration of 2,4-D as the auxin. However, callus was initiated much better on a variation of this medium containing a high level of 2,4-D, with or without the addition of three supplementary vitamins. Soft tissue formed in the presence of high 2,4-D or glycine, but became necrotic with repeated subculturing. Friable tissue was not obtained that would form viable cell suspensions and subsequent cell colonies on agar. Hard tissue, grown in liquid, produced very uniform tissue; and, when placed on Wolter's medium plus biotin, riboflavin, and calcium pantothenate, up to 65% of the pieces produced multiple aerial roots. Rooting was a function of the size and age of the explants. However, aerial roots did not differentiate into leafy shoots.

A friable agar culture was recovered for P. canescens that formed a cell-suspension in liquid medium. When cells of the suspension were poured onto agar nutrient, colonies of friable tissue were produced. No differentiation was observed. Rooting was obtained, however, on tissues of P. davidiana and P. grandidentata.

INTRODUCTION

Recent literature reviews have emphasized the importance of cell, tissue, and organ culture in the study of physiological problems in plants (1, 2). Of increasing interest is the use of this tool in forest research (3, 4). Meaningful experimental results can now be obtained within weeks by using tissue culture techniques (5), which may, in part, eliminate some of the difficulties of studying slow-growing trees.

Tree Research conducted at The Institute of Paper Chemistry has emphasized the improvement of Populus species for fast-growing, high-quality paper fibers. The naturally occurring triploid form of quaking aspen (P. tremuloides Michx.) has many desirable pulpwood characteristics (6) that makes this a high-interest species in our hybridization, polyploidy, and tissue culture programs.

The main objective of our tissue culture program is the production of genetically uniform trees for future breeding programs. In order to achieve this aim, numerous minor goals must first be attained, which necessarily will provide useful information for future studies of nutrition and physiology, as well as cell-wall and lignin formation. Several chemically defined media, required for the growth of quaking aspen tissue during various stages of this program, have been developed and are described in this report. Subsequent work will involve the establishment of liquid cell-suspension cultures and the differentiation of cell colonies into embryoids and independent plantlets.

Mathes (7), while at The Institute of Paper Chemistry, maintained hard and white tissue cultures of triploid quaking aspen on a medium containing major and minor elements, glycine, thiamine, coconut milk, naphthaleneacetic acid

(NAA), sucrose, and agar. Efforts to develop a defined medium were unsuccessful (8). However, the addition of 0.05% citric acid to the medium promoted root initiation, and the addition of NAA and kinetin produced occasional nonelongating leafy shoots (9).

Wolter (10, 11) developed a defined medium for diploid quaking aspen, ash, and pin oak. The inorganic nutrients were supplemented with myo-inositol, sodium--ferric--ethylenediaminetetraacetate chelate (Fe-EDTA), pyridoxine, nicotinic acid, thiamine, 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, sucrose, and agar. Inositol, thiamine, and an auxin were essential for aspen, but growth was stimulated by riboflavin, biotin, and calcium pantothenate. Some roots were initiated on aspen tissue grown on agar medium, but no shoots were formed.

The purpose of the present study was the development of chemically defined media that would grow the following types of triploid quaking aspen tissue.

On agar – 1. Hard white callus tissue
2. Hard white callus with root and shoot differentiation
3. Friable tissue

In liquid – 4. Hard white and uniform callus
5. Cell suspensions
6. Embryoid initiation and development

MATERIALS AND METHODS

Tissue cultures of triploid quaking aspen were started several times in 1964-5 by the root-shoot method (7). Preliminary tests showed no statistical difference between the growth of tissue recently isolated and tissue maintained since 1961 from Mathes' original isolation. Also, (a) regularly used deionized water was found to be as satisfactory for use in media as either distilled or double-distilled water, and (b) more tissue was produced in Petri dishes than in small closed bottles, such as those used by White and Risser (12).

Generally, 12-25 explants, weighing 10-20 to 30-50 mg. each, were distributed among 3-5 agar plates per treatment. Some of the tests chiefly measured qualitative traits and so were rated subjectively. In most tests, however, the fresh-weight (final minus initial) of individual pieces, as well as the growth factor (fresh weight/initial weight) was calculated. In liquid media all explants per flask were weighed together. For small flasks (250-ml. Erlenmeyer) 10 explants were grown in 50 ml. of medium in each of 3-5 flasks per treatment. In large flasks (3000 ml.) either 100 one-month-old explants or 50, 2 to 4-month-old spheres were grown in 500 ml. of medium. Both agar and liquid cultures were grown in the dark at 27°C. Agar plates were placed in a humid incubator, and flasks were placed on a rotary shaker at 150 r.p.m. Mathes' coconut-milk medium (No. 23) was used as the control, and a variation (medium 23E) supplied iron as Fe-EDTA instead of as iron citrate. The test media were autoclaved for 15 minutes at 15 p.s.i. after adjusting the pH to 5.5-5.8 with NH_4OH.

RESULTS

During the past two years, aspen tissue has been tested on over sixty chemically defined media. In many of the early tests, one or more vitamins were added to a basic medium, but, unfortunately, none of these were able to sustain vigorous white tissue growth of triploid quaking aspen. Many were, however, quite suitable for several other species of Populus and are described later in this report.

TRIPLOID QUAKING ASPEN TISSUE – AGAR MEDIA

After the appearance of Wolter's thesis (10), over thirty variations of his formula were tested in this laboratory. Wolter's revised medium is designated here as Medium 1 and contains a low concentration (0.04 mg./l.) of 2,4-D as the auxin. One of its variations, Medium W, is made with the high concentration (0.5 mg./l.) of 2,4-D found in Wolter's original medium. Media 2 to 5, described in Table I, are variations of Medium W. Media 11 to 15 are variations of Medium 1.

Test-A

Two experiments were performed in which the growth was compared between recently isolated tissue and callus initiated on root-shoot segments. In Media W-c and A-c the iron was supplied as iron-citrate rather than Fe-EDTA. At the end of each of three monthly subcultures, the average fresh weight in mg. was determined for each treatment. In some cases, the mean fresh weights between treatments were compared for significant differences (5% level) by Duncan's Multiple Test (13).

TABLE I

CHEMICALLY-DEFINED MEDIA

Component	Mg./l.	Medium																				
		W	1[a]	2	3	4a	4b	5a	5b	6	7	8	9	10	11	12	13	14	15	20	32	A
$MgSO_4$	764	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Na_2SO_4	425	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KNO_3	425	+	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$Ca(NO_3)_2$	170	+	+[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KCl	140	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaH_2PO_4	34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$MnSO_4$	9	+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
$ZnSO_4$	3.2	+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H_3BO_3	3.2	+	+	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
KI	1.6	+	+	+	+			+	+		+	+	+	+	+	+	+	+	+	+		
Fe-EDTA	5.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vitamin B_{12}	0.0015			+				+	+			+	+	+	+		+	+	+			
Biotin	0.01			+				+	+		+		+	+	+	+		+	+			
Ca-pantothenate	0.1			+				+	+		+	+		+	+	+	+		+			
Riboflavin	0.1			+				+	+		+	+	+		+	+	+	+				
Nicotinic acid	0.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Pyridoxine	0.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
Thiamine	0.1	+	+	+	+		+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Thiamine	0.01					+		+														
Glycine	0.7					+		+														
Glycine	6.7						+		+	+										+	+	+
$CuSO_4$	0.025									+										+	+	+
Na_2MoO_4	0.025									+										+	+	+
Inositol	100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2,4-D	0.04		+												+	+	+	+	+	+		
2,4-D	0.5	+		+		+	+	+	+	+	+	+	+	+								+
Kinetin	1.0		+												+	+	+	+	+	+		
NAA	5				+			+	+												+	
Sucrose	20,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Agar	8,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] Medium-1 is Wolter's revised medium.

The results given in Table II show that for all three passages, the growth of tissue on Media 2,4,A, and W nearly equalled or exceeded the growth on the control Medium 23. However, the fast-growing tissue was usually soft and yellow-tan in color and not as high in quality as the hard white tissue on Medium 1, W-c, and A-c. The quality of tissue in the first experiment remained fairly constant from the first passage (Fig. 1) through the second (Fig. 2) and third (not shown) passages. However, tissue quality declined appreciably in the second experiment from the first passage (Fig. 3) to the third (Fig. 4). The results were not conclusive as to whether Fe-EDTA or iron citrate was the best iron source.

Test-B

Experiment 1

Test A was repeated with some refinements. In the first experiment, a very uniform strain of triploid quaking aspen tissue was used that had been grown through ten subcultures from isolation on Medium W. This tissue was first grown on each of the test media for one or two subcultures, then explants (21 ± 4 mg.) were placed on each of ten defined agar media.

A two-way analysis of variance of individual fresh weights showed significant differences in growth between media, but no differences between replications and no interaction. When means were compared (Table III), fresh weights on Media 5b, 6, and W were significantly different from each other and all were greater than the control. However, the fast-growing tissue was soft and tan (Fig. 5). The best hard and white tissue grew on Media 1 and 3, on which 62% of the pieces rooted on the former (Fig. 6) and 25% on the latter.

TABLE II

TEST A RESULTS

Experiment 1. From tissue in fourth subculture on medium W

Passage 1	
2	469
A	423
W	315
4a	270
1	226
3	229
W-C	108
A-C	52
23	18

(See Fig.1)

Passage 2[a]	
23E	491
A	454
23	451
4a	446
2	404
W	392
3	300
5	190
1	143
W-C	137
A-C	122

($S_{\bar{x}}$ = 23)

(See Fig.2)

Passage 3	
2	706
W	643
23	548
23E	501
4a	491
A	334
A-C	197
3	128
1	58
W-C	42
5	20

($S_{\bar{x}}$ = 38)

Experiment 2. From root shoot segments. Passage 2 not shown

Passage 1 (initiation)
2
W
W-C
A
A-C
1
3
4a
23

(See Fig. 3)

Passage 3	
A	777
2	602
1	449
W	330
4a	289
23	202
A-C	72

(See Fig.4)

Passage 4	
W	684
A	566
4b	492
4a	484
23	471
2	427
1	357

[a]Average fresh-weights covered by the same vertical line were not different from each other, at the 5% level of significance, when compared by Duncan's Multiple Test.

Figure 1. Triploid Aspen Tissue Grown on Media 1, 2, 3, 4a (Top); 23, W, W-c, A, A-c (Bottom)

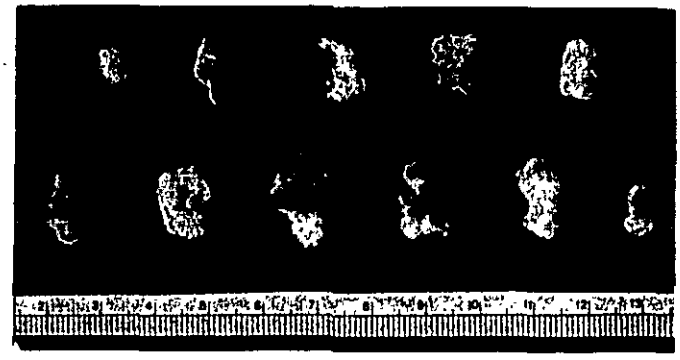


Figure 2. Media 1, 2, 3, 4a, 5 (Top); 23, 23E, W, W-c, A, A-c (Bottom)

Figure 3. Triploid Aspen Tissue Initiated on Media 1, 2, 3, 4a (Top); 23, W, W-c, A, A-c (Bottom)

Figure 4. Media 1, 2, 4a (Top); 23, W, A, W-c (Bottom)

TABLE III

TEST B, EXPERIMENT 1, PASSAGE 1

Medium	Fresh Weight	Growth Factor
5b	380	38
6	335	28
W	289	26
23	270	20
1	248	25
4b	240	24
A	240	22
4a	226	19
3	193	20
2	169	16
5a	4	0

($S_{\bar{x}}$=0.4)

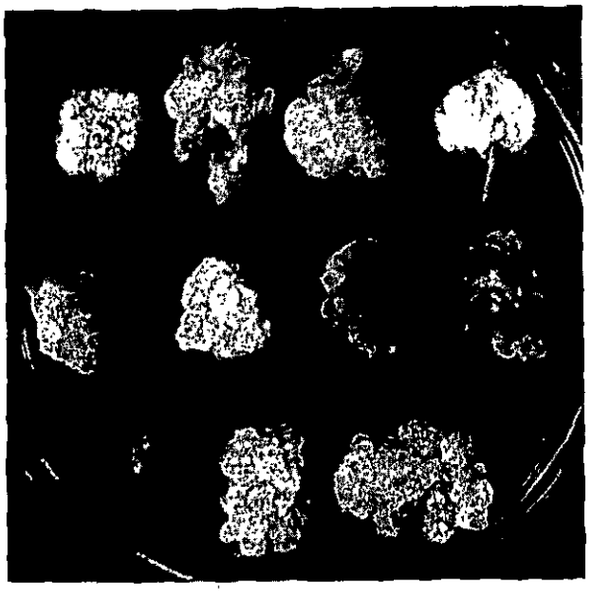


Figure 5. One Piece Each Grown on Media 23, W, A, 1 (Top); 2, 3, 4a, 4b, (Middle); 5a, 5b, 6 (Bottom)

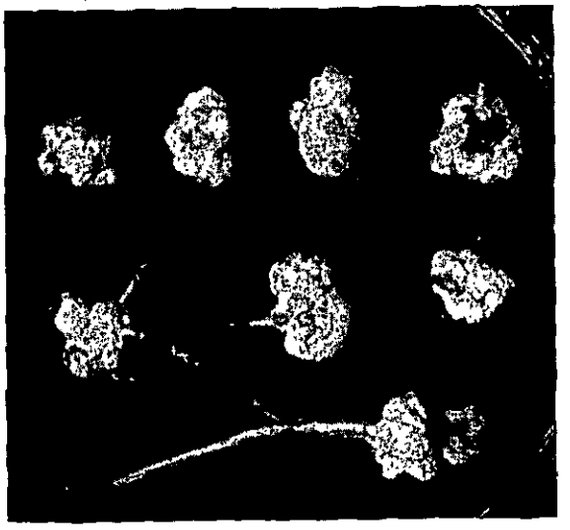


Figure 6. Medium 1, 62% Rooted Pieces

The best pieces of tissue on each medium of the first passage were subcultured onto fresh agar plates. After one month, 70% of the pieces on Medium 1 were rooted and 19% on Medium 3. Subsequent subcultures on Medium 3 led to increasing necrosis. However, the high rooting capacity was maintained on Medium 1 for several more subcultures.

Experiment 2

Root-shoot segments 5 mm.-long were placed horizontally on the surface of each test medium. After one month, the most callus was initiated on Medium A and the least on Medium 1 (Fig. 7). Hard white tissue occurred only on the controls and Medium 1. All other tissue was soft and tan. Tissue from all media was subcultured onto fresh agar plates for the second passage. After one month, only the tissue on four media (23, 23E, W, and A) was suitable for subculture (Fig. 8).

Explants (30 mg.) from Media 23, 23E, W, and A were placed on the fresh plates of the same medium as well as on Medium 1. Figure 9 shows that the subculture of tissue from Media W and A to Medium 1 stimulated the growth of hard white tissue. On the other hand, tissue left on each of these media during the third passage resulted in advanced necrosis. Rooting was stimulated in the transfer of tissue from Medium 23 to Medium 1, but inhibited from Medium 23E to Medium 1.

Test-C

The ability of citric acid to stimulate rooting was tested with a set of four modifications of Medium 1. In early tests, 2,4-D was included and inhibited rooting, so was eliminated. The auxin 3-indole acetic acid (IAA) was

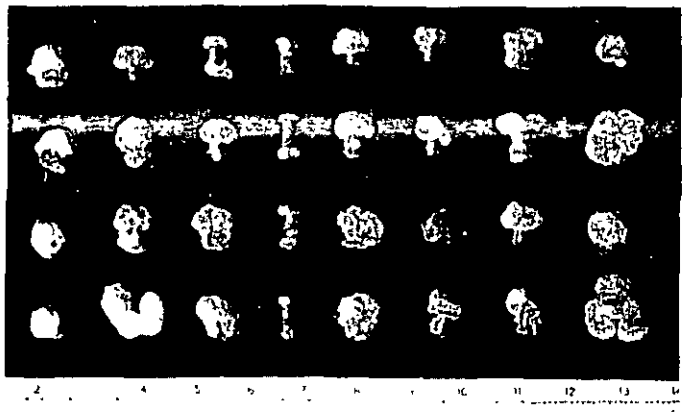


Figure 7. Vertical Rows from Left to Right, Media 23, 23E, W, 1, 2, 3, 5, A

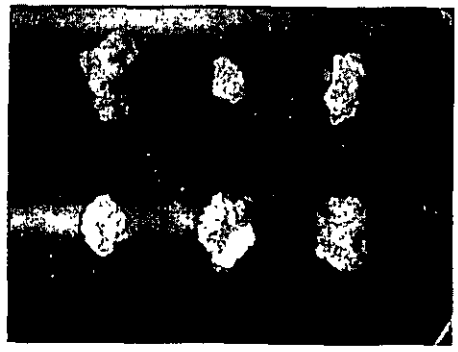


Figure 8. Media 2, 5, A (Top); 23, 23E, W (Bottom)

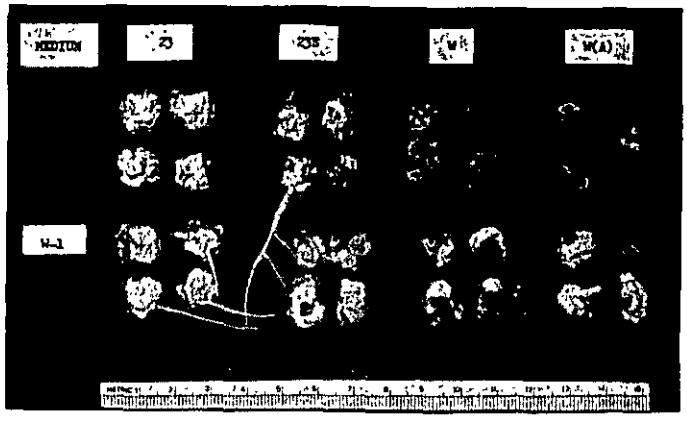


Figure 9. Top Two Rows, Grown on Original Medium. Bottom Two Rows, Tissue Transferred to Medium 1

present in all test media (Table IV) but not in Medium 1. Kinetin was added to Medium c and Medium d, and 0.05% citric acid [see Mathes (9)] added to Media b and d after autoclaving (to permit solidification).

After one month, 47% of the white and vigorous pieces on Medium 1 were rooted (Fig. 10) compared with only 20% on the less vigorous pieces on Medium b (containing citric acid).

TABLE IV

CITRIC ACID MEDIA

Medium	IAA, 2 mg./l.	Kinetin, 1 mg./l.	Citric Acid, 0.05%	Av. Fresh Weight, mg.	Percent Rooting
a	+			228	
b	+		+	99	20
c	+	+		160	
d	+	+	+	170	
1				472	47

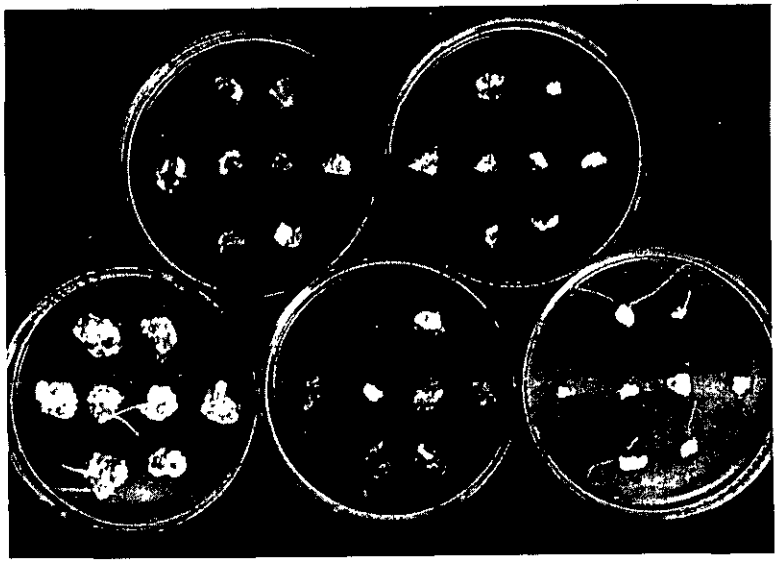


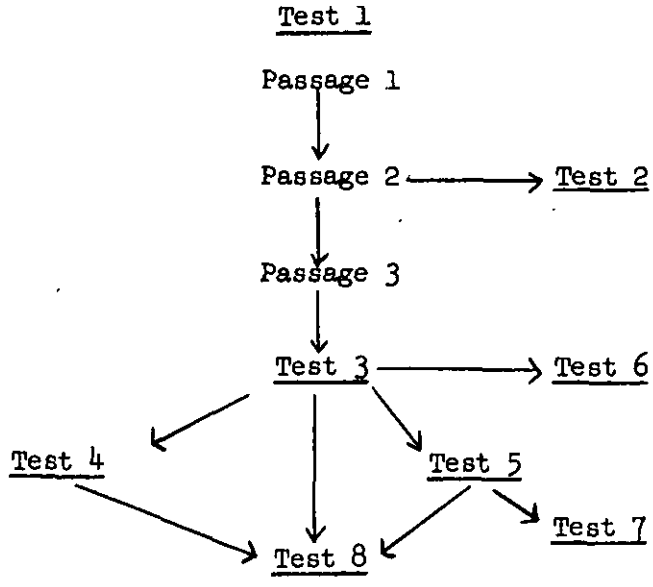
Figure 10. Citric Acid Test. Left to Right, Media c, d(Top); 1, a, b (Bottom)

TRIPLOID QUAKING ASPEN TISSUE – LIQUID MEDIA

A liquid culture consists of three parts, viz., the liquid nutrient medium, solid growing tissue, and a suspension of cells sloughed off of the solid tissue. In the following series of tests, all of the tissue originated from Test 1, which was derived from the same uniform triploid aspen culture used in agar-media Test B, Experiment 1. Tissue was carried from one test to another and occasionally subcultured several times within a test. The main sequence of these transfers is shown in Table V. Throughout this series, cell suspensions from the various tests were poured onto agar and semiliquid defined media. Unfortunately, no colonies of triploid aspen cells grew larger than 1-2 mm.

TABLE V

SEQUENCE OF LIQUID-MEDIA TESTS



Test 1

Tissue was grown in four 250-ml. flasks for each of five liquid test media. After one month, tissue was weighed and subcultured into fresh medium. This was repeated for two more passages. Growth was satisfactory in all media except Number 4 (Table VI). During the second (Fig. 11) and third (Fig. 12) subcultures, one-month-old spheres of tissue in Medium 1 became increasingly lighter yellow in color and more uniform in appearance than tissue in any other medium.

Test 2

Spheres of tissue growing in Media W, 1, and 2 in Test 1, at the end of the second passage, were cut into eight pieces each (1/8-sphere explants) and placed in 3-liter flasks. After one month, spheres were transferred intact to fresh media for one more month. The two-month-old spheres averaged 890 mg. in Medium W, 667 mg. in Medium 2, and 370 mg. in Medium 1. However, the spheres in Medium 1 were lighter yellow in color and more uniform in size than in the other two media (Fig. 13).

Test 3

From the third passage of Test 1, 25 one-month-old spheres were cut into 1/8-explants and grown in Medium 1 in large flasks. After one month, the average weight per sphere increased from 34 to 297 mg., for a fresh weight of 261 mg. and a growth factor of 8 (Fig. 14). This tissue was light yellow and uniform, and was used in Tests 4 to 6. Monthly subcultures of one-month-old spheres were also started.

TABLE VI

LIQUID TEST 1[a]

Passage 1			Passage 2			Passage 3		
W	253	22	2	225	8	1	249	9
1	252	21	W	218	8	2	218	8
3	200	16	3	182	6	W	213	8
4a	164	11	1	157	6	3	174	6
2	154	14	4a	105	4	4a	118	5
			(See Fig. 11)			(See Fig. 12)		

[a]For each passage is given the medium, the average fresh weight in mg., and the growth factor. Initial weights of explants were, respectively, ca. 13, 28, and 27 mg.; and the $S_{\bar{x}}$ for each passage was 13 mg.

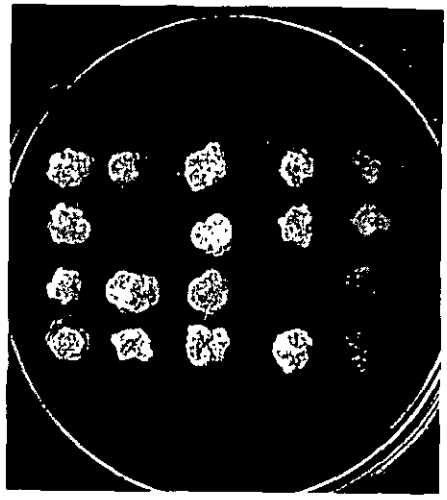


Figure 11. Vertical Rows From Left to Right, Media W, 1, 2, 3, 4a

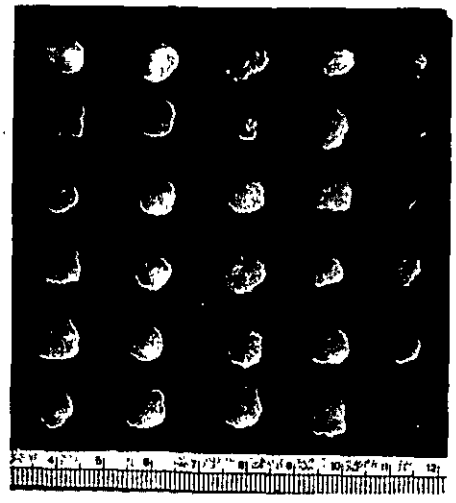


Figure 12. Vertical Rows From Left to Right, Media W, 1, 2, 3, 4a

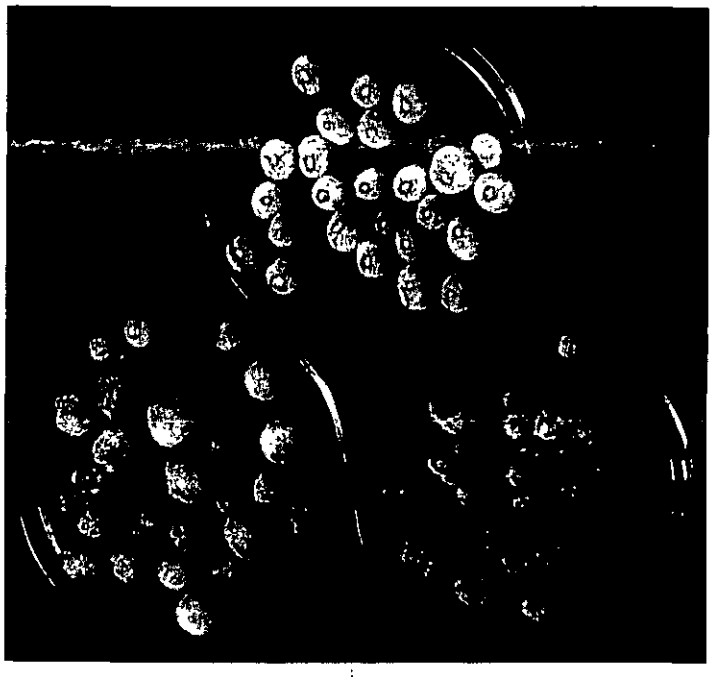


Figure 13. Media 1 (Top), W (Left), 2 (Right)

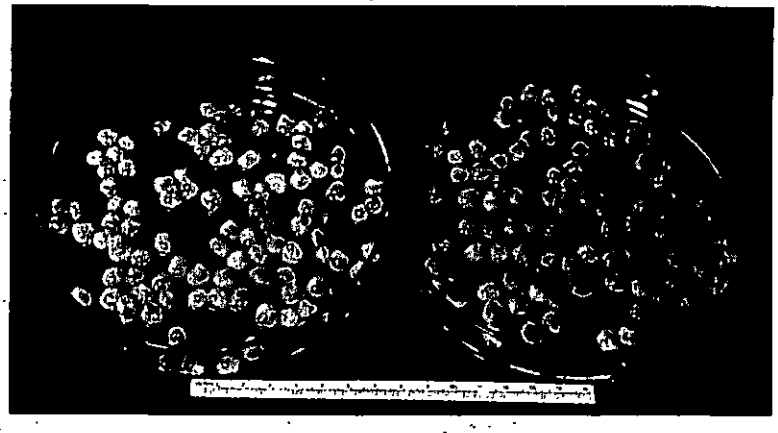


Figure 14. One-Month-Old Tissue Grown in Medium 1

Test 4

Fifty spheres from Test 3 were transferred directly to fresh Medium 1 for one month. As two-month-old spheres, their weight increased from 300 to 440 mg., for a growth factor of 1.5. This tissue was grown for another month in fresh medium and used in Test 8 as three-month-old spheres.

Test 5

One hundred 1/8-sphere explants from Test 3 were grown for one month in Medium 1 in large flasks. The average weight increase was from 47 to 380 mg., for a fresh weight of 330 mg. and a growth factor of 7. This tissue was used in Test 7.

Test 6

Tissue from Test 3 was cut into eighth, quarter, and half-sphere explants and some were left whole. Explants of each size were placed on agar Medium 1 for one month. The smallest explants (1/8-spheres) predictably had the highest growth factor (12). However, the greatest average fresh-weight (725 mg.), as well as the most usable-white-tissue (312 mg.) was found on the tissue grown from the 1/2-sphere explants (Table VII).

TABLE VII

VARIABLE-SIZED EXPLANTS GROWN ON AGAR MEDIUM 1

Explant Size	Initial Weight	Fresh Weight	Growth Factor	Usable Tissue Weight	Usable Tissue Percent
Whole spheres	330	715	2	208	29
Half spheres	154	725	5	315	43
Quarter spheres	93	518	6	225	44
Eighth spheres	34	518	12	200	38

Test 7

Experiment 1

Tissue from Test 5 was cut into 1/4-spheres (ca. 90 mg. each) and 18 explants distributed among three agar plates each of 19 media. Uniformly hard and white tissue grew vigorously on most of the media (Fig. 15, Table VIII).

However, no roots were initiated on tissue grown on media containing a high concentration (0.5 mg./l.) of 2,4-D (Media W, 2, 4 to 10). Multiple, vigorous roots were found on 11 to 67% of the pieces grown on Media 1, 11 to 15, and 21 which contained a low concentration of 2,4-D (0.04 mg./l.) and kinetin. Roots also occurred on Medium 3 tissue containing NAA as the auxin. In this study, when four vitamins were added to Medium 1, rooting was inhibited by vitamin B_{12} but stimulated by biotin, calcium pantothenate, and riboflavin (Fig. 16).

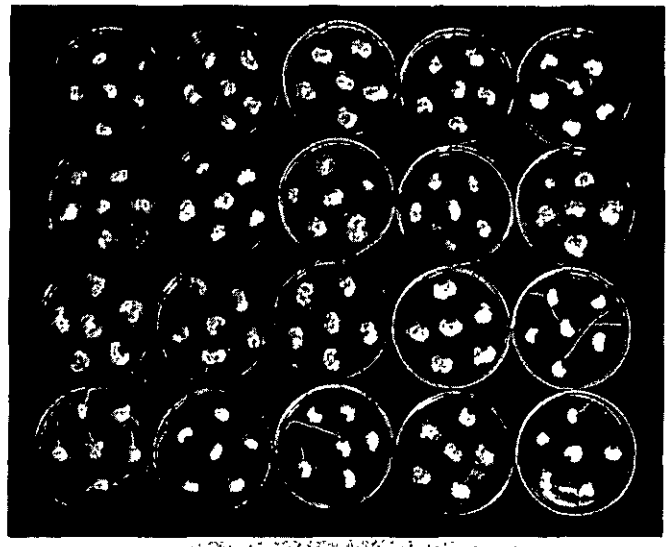


Figure 15. One Plate per Medium. From Left to Right (Top Row), Media 23 New, 23 Old, 23 E, W, 1; (Second Row) 2, 3, 4b, 5b, 6; (Third Row) 7, 8, 9, 10, 11; (Bottom Row) 12, 13, 14, 15, 21

TABLE VIII

TEST 7, EXPERIMENT 1[a,b]

Low 2,4-D			NAA			High 2,4-D	
1	410	22	23 new	356		W	543
11	295	39	23 old	525		2	490
12	382	67	23 E	707		4b	450
13	303	6	3	471	6	5b	328
14	425	33				6	642
15	562	11				7	769
21	364	22				8	521
						9	492
						10	551

[a] Medium, average fresh weight in mg., and percent rooted pieces are given.

[b] All tissue was hard and white except for the soft yellow tissue on Media 4b and 5b.

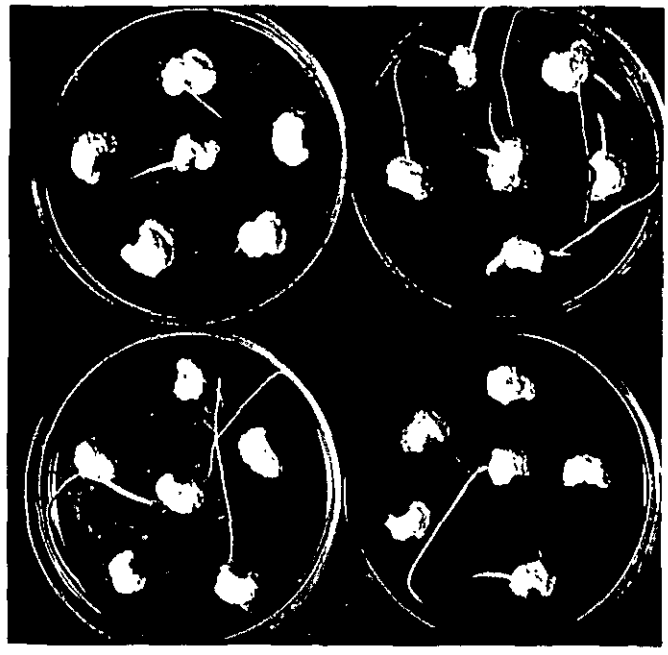


Figure 16. Left to Right (Top) Media 1, 12; (Bottom) 11, 14

Experiment 2

Four 20-60 mg. explants, subcultured from tissue in the first experiment of Test 7, were placed on each of two plates per medium. Fastest growth occurred on Medium 7, but was soft and tan as in the first passage (Fig. 17, Table IX). The high quality of the fast-growing tissue on Medium 10 was still evident. Twelve percent of the pieces on Medium 11 were rooted, 50% on Medium 14, and 62% on Medium 13 (Fig. 18). No roots were found on Medium 1, or on Medium 12 which had the most roots in Experiment 1. In this case, vitamin B_{12} did not inhibit the initiation of roots

Experiment 3

From the second experiment, 10 explants from the tissue on Media 1, 11, and 12 were grown on each of two agar plates. After one month, 15 and 20% of the pieces on Media 11 and 12, respectively, had rooted. No roots were initiated on tissue on Medium 1.

Test 8

Spheres of tissue one, two, and three months old, from Tests 3, 4, and 5, respectively, were cut into 1/8-sphere explants. After 17 days' growth in liquid Medium 1, the average sphere weighed 180, 275, and 260 mg. for the three age classes. This tissue was then cut into 1/8 and 1/4-explants (Fig. 19), and 100 explants of each size distributed among 20 agar plates of Medium 12 for each age class. Twenty pieces from each treatment were weighed individually at the beginning and end of the experiment.

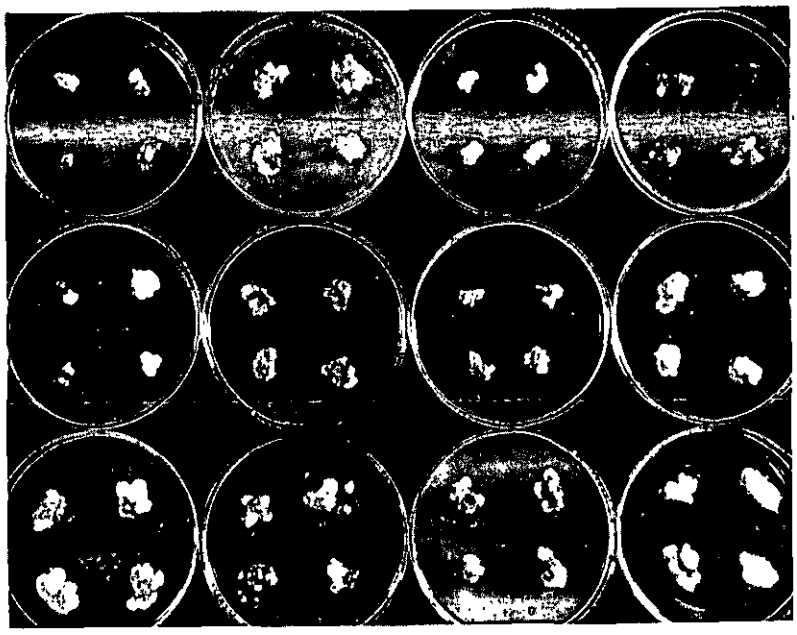


Figure 17. From Left to Right (Top Row) Media 23, W, 1, 2; (Middle) 3, 4b-White, 4b-Friable, 5b; (Bottom) 7, 8, 9, 10

TABLE IX

TEST 7, EXPERIMENT 2

Medium	Average Fresh Weight	Growth Factor	Percent Rooting
7	1024	23	
10	946	27	
5b	712	13	
W	712	12	
9	590	14	
8	527	12	
11	405	11	12
3	394	8	
14	391	9	50
12	368	9	
4b-friable	306	6	
1	289	5	
4b-white	270	7	
13	269	8	62
2	262	6	
23	188	5	

($S_{\bar{x}}$ = 27)

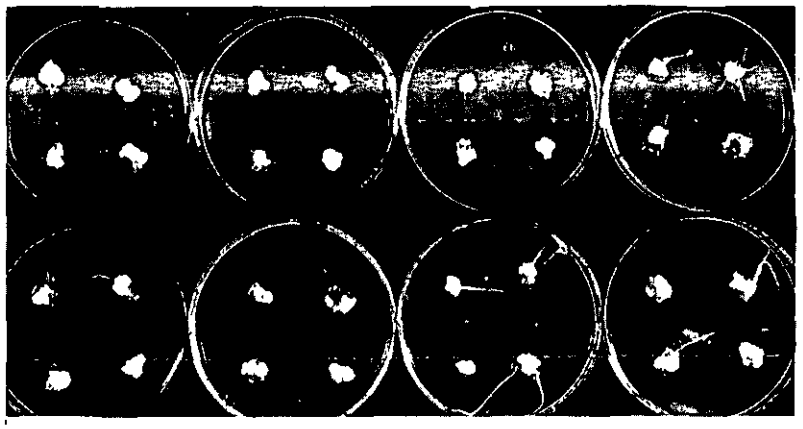


Figure 18. From Left to Right (Top and Bottom) Media 11, 12, 13, 14

Explant Size	Age, months 1	2	3
1/8	A-1	A-2	A-3
1/4	B-1	B-2	B-3

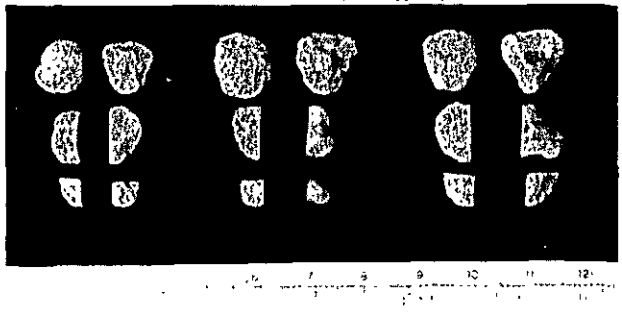


Figure 19. Spheres (Left to Right) from One, Two, and Three-Month-Old Cultures Cut into Half, Quarter, and Eighth-Sphere Explants

The largest average fresh weight (652 mg.) was found on 3-month-old tissue (B-3) from 1/4-sphere explants (Table X). Tissue B-3 had twice as many rooted pieces (65%) as the same-aged tissue (A-3) cut as 1/8-explants, as well as twice as many rooted pieces as the same-sized explants (B-1) of one-month-old tissue (Fig. 20). Clearly, both the age and size of the explants influenced their rooting ability. Hard white tissue began growing in all treatments four days after the start of the experiment. Many rooted pieces had 3-5 roots per piece, some of which grew on or into the agar, but mostly as aerial roots in various degrees of erectness (Fig. 21).

From each treatment, 14 rooted and 14 unrooted pieces were distributed among four agar plates of Medium 12. After two weeks, 71% of the unrooted B-3 pieces had initiated roots (chiefly aerial) with a corresponding increase in rooting (Fig. 21) for all other age and size classes (Table XI). The most vigorous and well-rooted pieces from all cultures were then transferred to fresh Medium 12 for three more monthly passages. However, none of the aerial roots differentiated into leafy shoots, either in the dark or under strong artificial illumination.

Test 9

In order to test the effects of a high level of phosphorous and potassium on rooting, 40 1/8-sphere explants were distributed among 10 agar plates each of Media 12, 12+10xP, and 12+10xK. After one month, 42% of the pieces on Medium 12 and 35% on Medium 12(P) were rooted, but no roots were observed on Medium 12(K) tissue. Vigor and rooting ability were both depressed by the addition of 10x phosphorous and potassium (Fig. 22). However, some interesting green-friable, as well as hard-white pieces of tissue were found on Medium 12(K).

TABLE X

TEST 8 RESULTS

Initial		Final			
Explants	Fresh-Weight	Tissue	Fresh-Weight	Growth Factor	Percent Rooting
B-3	133	B-3	652	5	65
B-2	129	A-3	574	6	49
B-1	107	B-2	498	4	31
A-3	88	A-1	355	5	37
A-2	81	A-2	345	4	46
A-1	70	B-1	248	2	33
	($S_{\bar{x}} = 4$)		($S_{\bar{x}} = 24$)		

Figure 20. Rooted Tissue After One Month on Medium 12. From Left to Right, Tissue A-1, A-2, A-3 (Top); Tissue B-1, B-2, B-3 (Bottom)

TABLE XI

TEST 8

Tissue	New Roots, %
B-3	71
A-3	43
A-2	29
B-1	29
A-1	21
B-2	21

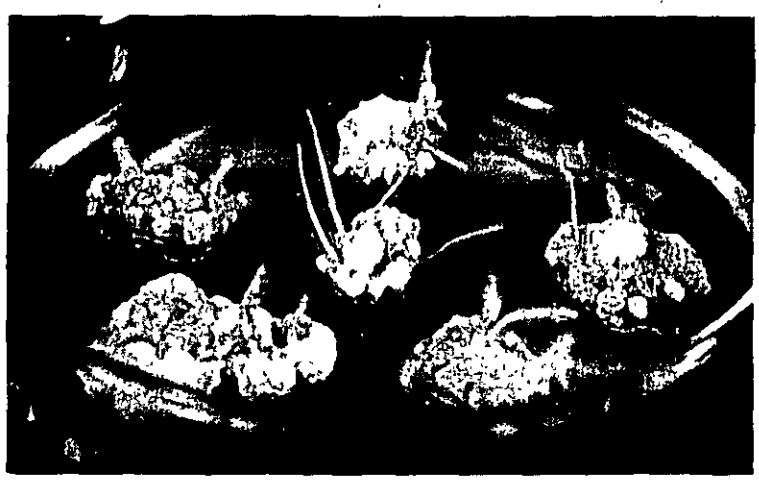


Figure 21. Aerial Roots on Tissue Grown for Two Months on Agar Medium 12

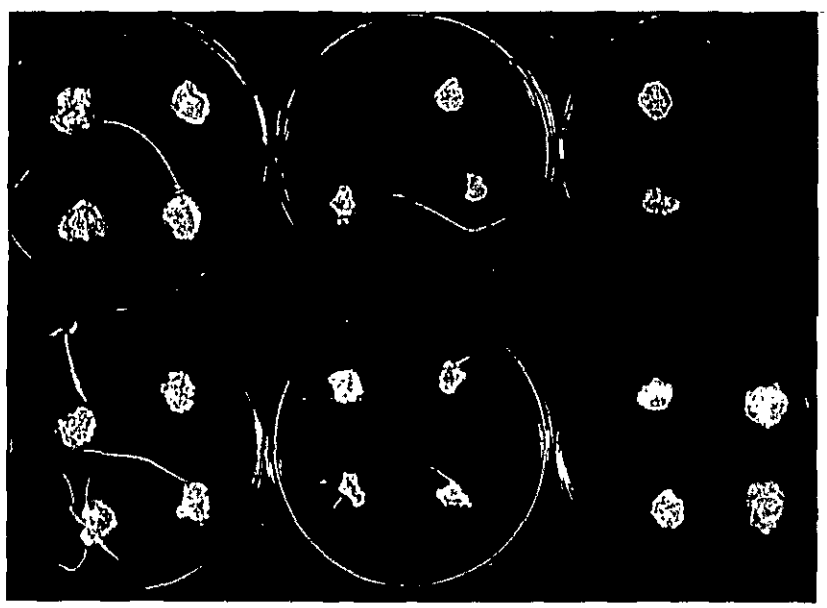


Figure 22. Left to Right (Top and Bottom) Media 12, 12(P), 12(K)

Test 10

A new series of liquid cultures was started from explants of Test 8. in order to recheck the results of Test 8. After four months, four age classes of tissue were growing with the average weight of the spheres being (1) 230 mg., (2) 700 mg., (3) 1000 mg., and (4) 1300 mg. (Fig. 23). Unfortunately, all cultures became contaminated during the summer of 1966 and were discarded.

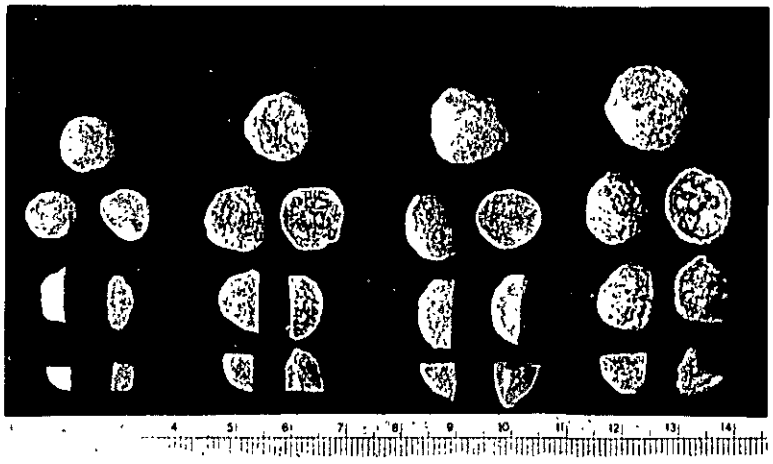


Figure 23. Left to Right, Tissue Grown in Liquid Medium 12 for 1, 2, 3, and 4 Months

OTHER SPECIES OF POPULUS

In 1963, Mathes (8) isolated callus tissue onto his coconut-milk Medium 23 from white poplar (Populus alba L.), eastern cottonwood (P. deltoides Marsh.), gray poplar [P. canescens (Ait.) Sm.], bigtooth aspen (P. grandidentata Michx.), and the Chinese variety of European aspen [P. tremula var. Davidiana (Dode) Schneid.] hereafter referred to as P. davidiana. In late 1964, these

cultures were transferred to Medium 23E, and subsequently used to test defined media concurrently with tissue from triploid quaking aspen.

In the early studies, before variations of Wolter's medium were tested, ten vitamins were added to the basic Medium Number 32 (see Table I) in three series, viz., (1) one vitamin at a time, (2) one to three sets of 3-4 vitamins each, and (3) all except one vitamin at a time. The auxin in all media was NAA at 5 mg./l., and the vitamins are given in Table XII.

TABLE XII

VITAMINS USED IN DEFINED MEDIA

Vitamin	Mg./l.
Group-A	
Nicotinic acid	0.5
Pyridoxine	0.1
Hypoxanthine	25
Group-B	
Biotin	0.01
Calcium pantothenate	0.1
Choline chloride	10
Group-C	
Ascorbic acid	0.1
Sorbitol	10
Riboflavin	0.1
Vitamin B_{12}	0.0015

Tissue of triploid P. tremuloides, P. alba, and P. deltoides did not form white and vigorous tissue on any of the 35 vitamin media tested. Promising cultures of the other species were carried along through several subcultures on agar media, and the best results are given in Table XIII.

TABLE XIII

DEFINED MEDIA FOR SPECIES OF POPULUS[a]

P. canescens	Hard-white vigorous	Agar 48
	Friable white vigorous	W, 1
P. davidiana	Hard-white rooted	63, 66
	Friable yellow	W
P. grandidentata	Soft yellow rooted	61
P. tremuloides, 3X	Hard white: callus initiation	W, 10
	subculture	1
	rooting	11-14
	Soft light-green vigorous	10
	Friable yellow-tan	4b
P. canescens	Cell suspension	Liquid W
P. tremuloides, 3X	Hard light-yellow	1

Composition	Medium
[a]Medium-32 plus: Groups A and B (Table XIII)	48
All vitamins except-ascorbic acid	61
choline chloride	63
sorbitol	66

The tissue of P. canescens grew better on a greater variety of media than did any other species. Hard-white callus consistently grew vigorously on Medium 48, and later, on Medium W. Some pieces on Medium W became friable and formed a viable cell-suspension when placed in liquid Medium W (Fig. 24). When part of the cell-suspension was poured back onto agar Medium W, fast-growing colonies of white and completely friable tissue were recovered (Fig. 25). Also recovered were rooted pieces of P. davidiana on Media 63 and 66, and P. grandidentata on Medium 61 (Fig. 26).

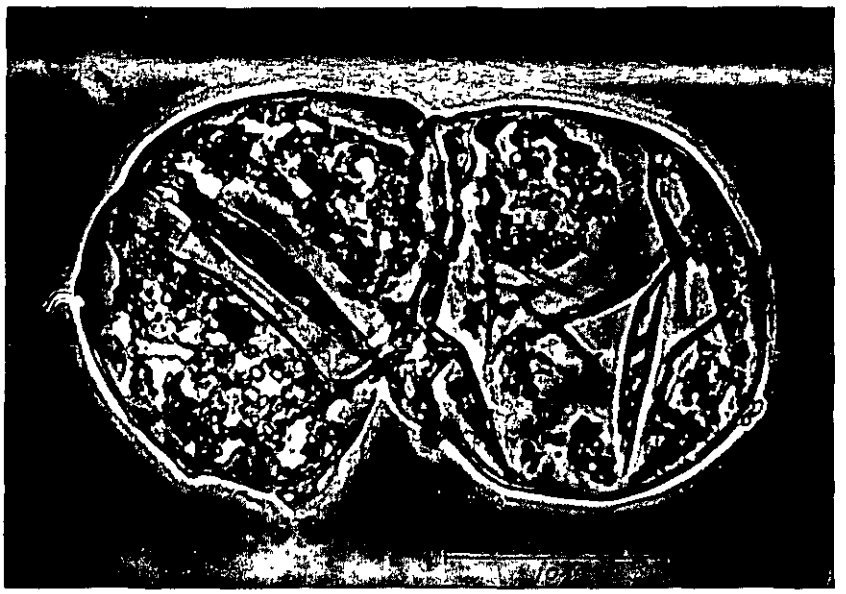


Figure 24. A Three-Celled Colony Derived from One Cell of Friable *Populus canescens* Tissue in Liquid Medium W

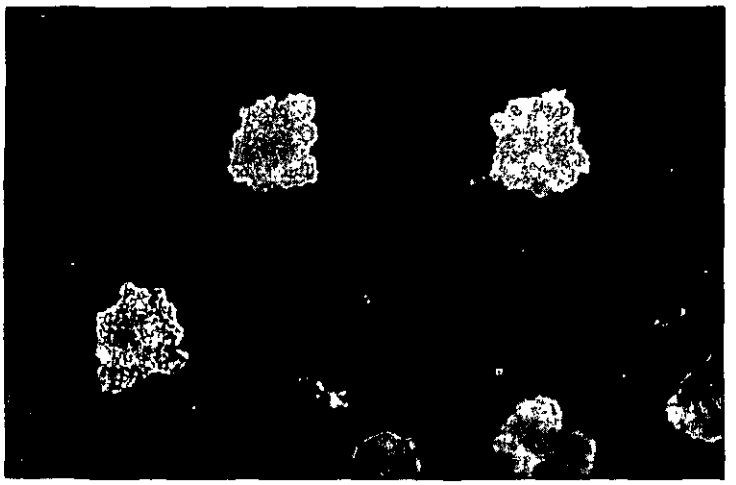


Figure 25. Friable Agar Culture of *Populus canescens*

Figure 26. Rooted Pieces of Populus grandidentata on Medium 61 (Top) and P. davidiana (Bottom) on Medium 63 (Left) and 66 (Right)

In later tests, P. davidiana tissue from Medium W grew soft and white on Medium 1, but friable and tan when returned to Medium W (Fig. 27). On the other hand, P. canescens tissue from Medium 48 did not grow as well on Medium 1 as when left on Medium 48 (Fig. 28).

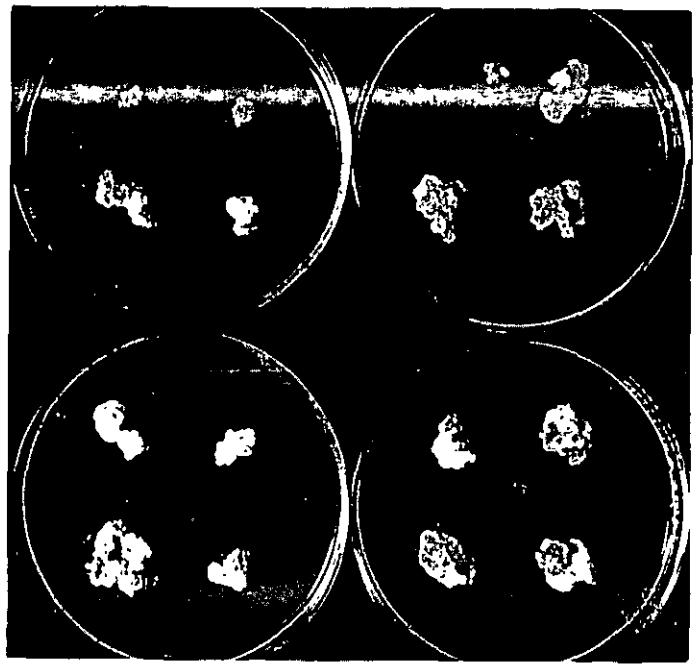


Figure 27. P. davidiana. From Medium W: Subcultured to Medium 1 Twice (Top and Bottom Left); to Medium 1 then Medium W (Top Right); and to Medium 1 Three Times (Bottom Right)

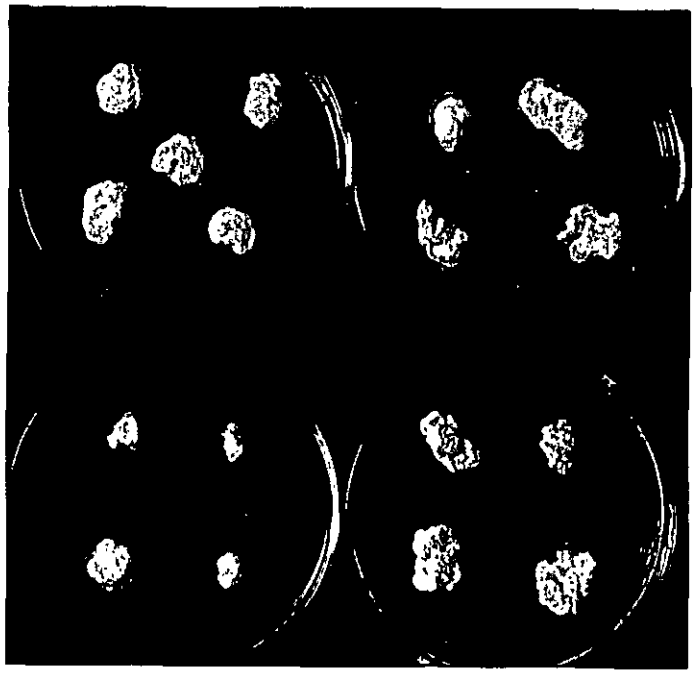


Figure 28. P. canescens. From Medium 48: Subcultured to Medium 23 (Top Right), Medium 1 (Bottom Left and Right), and Medium 48 (Top Left)

DISCUSSION

Several of the defined media necessary for our tissue-culture program are now available for the study of triploid quaking aspen, as well as for P. canescens and P. davidiana. The revised medium proposed by Wolter (10), designated in this report as Medium 1, produced hard white and vigorous growth of triploid aspen tissue on agar, with up to 20% rooting. In liquid, light yellow and very uniform spheres were formed.

Callus was poorly initiated on root-shoot sprouts on Medium 1, but was greatly enhanced when the high concentration of 2,4-D was used as given in Wolter's original formula (Medium W). Perhaps during initiation, much higher amounts of auxin are required than later, during subculturing, when the tissue itself may produce a limited amount of natural auxin. The synergistic effects of kinetin on low levels of 2,4-D (as observed by Wolter) may also be different during initiation than when tissue is vigorously reproducing itself. In this study, tissue initiated on a variety of media always did better when subcultured to Medium 1.

On the other hand, friable tissue was not found on Medium 1. When callus, initiated on the high – 2,4-D Medium W, was repeatedly subcultured the tissue became soft but also often tan or necrotic. The high auxin concentration thus appears to have a positive effect on friability. The same results are seen when glycine was present such as in Media 4, 5, 6, and A. When vitamin B_{12}, biotin, and calcium pantothenate were added to Medium W to give Medium 10, soft callus was initiated that was light green in color although grown in the dark. After repeated subcultures, the tissue on Medium 10 became white but remained soft. The vitamins, in this case, appear to have inhibited or counteracted the effects of the high auxin concentration.

One interesting aspect of this study was the difference in rooting ability exhibited on agar, by liquid-grown triploid tissue, according to the size and age of the explant. The increased rooting with both size and age could mean that the larger and older pieces have both a larger reservoir of metabolites, as well as better differentiated tissue. Spheres of different ages were collected in fixative for a later analysis for differentiation in prepared cross sections.

FUTURE PLANS

Our main objective now is to develop a medium which will give a truly friable culture of triploid aspen. Given a friable culture, we would then progress toward maintaining two types of viable cell-suspensions that would (1) permit continuous, undifferentiated growth and (2) initiate embryoid differentiation in liquid. Advanced embryoids probably would grow normally if placed on the surface of agar Medium 12 developed during this study.

Work will also proceed with other species, particularly P. canescens, in which the friable-culture, cell-suspension, friable-culture cycle has already occurred. For this species, media will be sought to stimulate differentiation.

ACKNOWLEDGMENTS

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